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Characterization of metallothioneins (MT-I and MT-II) in the yak[1]

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ABSTRACT: The cDNA-encoding sequences for yak metallothionein isoforms I (MT-I) and II (MT-II) were amplified and cloned by reverse-transcription PCR to characterize the nucleotide sequence and protein structure of metallothionein in the yak. The cDNA sequences of MT-I and MT-II were subjected to BLAST searching at the National Center for Biotechnology Information, and the results indicated that the nucleotide sequences of yak MT-I and MT-II, when compared among different species of mammals, are highly conserved. The yak open reading frames have a length of 183 nucleotides, which encode for yak MT-I and MT-II proteins of 61 AA, respectively. Analysis of hydrophobicity, transmembrane region, and signal peptides suggested that metallothioneins of the yak are nonsecretory proteins. There were several conserved tripeptide sequences, such as C-X-C, C-C-X-C-C, and C-X-X-C (X designates AA excluding cysteine in MT-I and MT-II), and they are highly conserved in their evolution. By homologous comparative modeling, we predicted the molecular spatial structures of yak MT-I and MT-II, which are composed of α- and β-domains that are linked by the conserved tripeptide Lys^{30}-Lys^{31}-Ser^{32} (KKS).

Key words: cDNA, characterization, metallothioneins, structure, yak

INTRODUCTION

Metallothioneins (**MT**) are low-molecular-weight (approximately 7,000 Da) and cysteine-rich, metal-binding proteins that are found in microorganisms, plants, and animals (Margoshes and Vallee, 1957; Luce et al., 1993; Syring et al., 2000). Metallothionein was first isolated as a cadmium-binding protein from horse kidney (Margoshes and Vallee, 1957). Metallothioneins have an unusual AA composition in that there are no aromatic AA in the chemical structure. The single polypeptide chain of MT is composed of 61 AA, in which cysteine comprises 33% of its total residues.

Metallothioneins are believed to play a central role in regulating tissue concentrations of essential metals (e.g., Zn and Cu) and to be involved in detoxification of toxic metals (e.g., Cd and Hg; Kägi, 1991; Roesijadi, 1992, 1996). The MT multigene family is composed of at least 4 isoforms: MT-I, MT-II, MT-III, and MT-IV (Nordberg and Nordberg, 2000). Metallothionein-I and MT-II exist in all tissues (Searle et al., 1984; Hamer, 1986). Metallothionein-III is expressed mainly in the brain (Palmiter et al., 1992), and MT-IV is expressed in stratified squamous epithelium (Quaife et al., 1994). Heavy metals, hormones, inflammation, acute stress, and many chemicals can induce MT-I and MT-II activity.

The yak species (*Bos grunniens*) represents a unique bovine species adapted to the Qinghai-Tibetan plateau of China at altitudes of 3,000 m above sea level. Yak are usually the dominant species in such environments, which are ill-suited for cattle or sheep (Zhang et al., 1994). Yaks are used for meat, milk, draft, and fiber and are a major economic resource for herders in this region. Because the Qinghai-Tibetan plateau is subject to pollution from heavy metal contaminants, there is a need to evaluate the isoforms of MT in this species to aid in the determination of potential tolerance to these contaminants. Thus, the objective of this research was to charac-

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terize the nucleotide sequence and structure of MT-I and MT-II in the yak.

MATERIALS AND METHODS

Animals and RNA Isolation

Preharvest animal care and use was under control of local farmers in Gannan, Gansu production area and was consistent with Gansu Agricultural University animal care and use requirements. Animals were harvested at a commercial facility that must comply with state regulations governing processing of meat animals. To clone the MT genes, liver samples were obtained from 25 domestic yaks (*B. grunniens*) from Gannan, Gansu, China, within 10 min after slaughter. The samples were flash-frozen in liquid nitrogen and stored at –80°C until thawed for RNA extraction. Total RNA was extracted with Trizol reagent (Gibco BRL, Gaithersburg, MD). Yak liver tissue samples were homogenized in 1 mL of Trizol reagent per 50 to 100 mg of tissue using a glass homogenizer. Homogenized samples were incubated for 5 min at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Chloroform (0.2 mL/mL of Trizol) was added, and samples were shaken and incubated at 15 to 30°C for an additional 2 to 3 min. Samples were then centrifuged at $12{,}000 \times g$ for 15 min at 2 to 8°C. After centrifugation, the dissolved RNA was pipetted to a fresh tube, and the RNA was precipitated with isopropyl alcohol. Samples were then incubated at 15 to 30°C for 10 min and centrifuged at $12{,}000 \times g$ for 10 min at 2 to 8°C. The supernatant was removed, and the RNA precipitate was washed with 75% ethanol. The RNA and ethanol were vortexed and centrifuged at $7{,}500 \times g$ for 5 min at 2 to 8°C. The RNA was then redissolved in 100% formamide (deionized) and stored at –70°C.

Reverse-Transcription PCR Primers

The oligonucleotide primers for reverse-transcription PCR (**RT-PCR**) were designed based on the coding region sequences of MT-I and MT-II in the cow (*Bos taurus*), sheep (*Ovis aries*), mouse (*Mus musculus*), rabbit (*Oryctolagus cuniculus*), and pig (*Sus scrofa*) published at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/; last accessed Dec. 6, 2006). The forward and reverse primers, respectively, of the yak MT-I and MT-II were $Y_{MT}SP_1$ (21 bp): 5′-ATGGACCCGAACTSCTCCTGC-3′; and $Y_{MT}SP_2$ (23 bp): 5′-GGCRCAGCAGCTGCACTTGTCCG-3′.

RT- PCR

The RT-PCR was carried out using an RNA PCR kit, Version 2.1 (TaKaRa, Shiga, Japan), for which avian myeloblastosis virus reverse transcription was used for the first-strand DNA synthesis, and *Taq* DNA polymerase was used for PCR in a single optimized RT-PCR buffer. First-strand cDNA synthesis was accomplished

Table 1. Reaction parameters for the Touch Down PCR

Reaction process	Reaction temperature and time			Reaction cycles
	Denaturing	Annealing	Renaturing	
1	94°C (30 s)	58 (1 min)	72°C (2 min)	5
2	94°C (30 s)	56 (1 min)	72°C (2 min)	5
3	94°C (30 s)	54 (1 min)	72°C (2 min)	5
4	94°C (30 s)	52 (1 min)	72°C (2 min)	10
5	94°C (30 s)	50 (1 min)	72°C (2 min)	15

by RT-PCR at 30°C for 10 min, 50°C for 30 min, 99°C for 5 min, and 5°C for 5 min, and amplification was carried out by Touch Down PCR (Don et al., 1991) for 40 cycles, as described in Table 1.

Products were separated by electrophoresis on a 1.5% agarose gel. Fragments with the expected size were cut from the gel and purified using the PCR Preps DNA Purification System (Promega, Madison, WI).

Cloning and Sequencing of cDNA Encoding the MT-I and MT-II Genes

The plasmid library was constructed with T4 polymerase by ligating the amplified DNA fragments into the *Eco*RV and *Hind*III site of the cloning plasmid pZeroT-Vector (Cat. no. K2600-01, Invitrogen, Shanghai, China), following the manufacturer's procedures. Isolation of library DNA, transfection of competent *Escherichia coli* DH5α, and extraction of DNA from transfected cells were performed according to published methods (Sambrook and Russell, 2001). Recombinant plasmids containing relevant yak DNA fragments were sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

Sequence Analysis of the Nucleotides and AA

The nucleotide and AA sequences of MT-I and MT-II in the yak were subjected to BLAST searching at the National Center for Biotechnology Information. Multiple comparisons of the nucleotide sequences were then performed using BioEdit (http://www.mbio.ncsu.edu/; last accessed Dec. 6, 2006). Characterization of MT-I and MT-II included determination of their molecular weight, AA composition, hydrophobicity, transmembrane region characteristics, and signal peptide analysis.

Hydrophobicity was analyzed using ProtScale and ExPASy, and evidence for transmembrane regions was analyzed using ExPASy (prediction parameters: TM-helix length between 17 and 33). Parameters used in the ProtScale analyses included a window size of 9, window weight on the edges of 100%, the linear weight variation model, and no normalization of the scale. Protein signal peptides were analyzed using SignalP 3.0 software (Bendtsen et al., 2004; http://www.cbs.dtu.dk/services/SignalP-3.0/; last accessed Dec. 6, 2006). Analyses used neural networks and hidden Markov models trained on eukaryotes. Secondary structures of MT-I and MT-II were

YAK. MT– I

1	ATG	GAC	CCG	AAC	TGC	TCC	TGC	TCC	ACT	GGC	GGC	TCC	TGC	AGC	TGC
1	M	D	P	N	C	S	C	S	T	G	G	S	C	S	C
46	CCT	GGC	TCC	TGC	ACC	TGC	AAG	GCC	TGC	AGA	TGT	CCC	TCC	TGC	AAG
16	P	G	S	C	T	C	K	A	C	R	C	P	S	C	K
91	AAG	AGC	TGC	TGC	TCC	TGC	TGC	CCT	GTG	GGC	TGT	GCC	AAG	TGT	GCC
31	K	S	C	C	S	C	C	P	V	G	C	A	K	C	A
136	CAG	GGC	TGC	ATC	TGC	AAA	GGG	GCC	TCG	GAC	AAG	TGC	AGC	TGC	TGT
46	Q	G	C	I	C	K	G	A	S	D	K	C	S	C	C
181	GCC														
61	A														

YAK. MT– II

1	ATG	GAC	CCG	AAC	TGC	TCC	TGC	ACC	GCG	GGT	GAA	TCC	TGC	ACA	TGT
1	M	D	P	N	C	S	C	T	A	G	E	S	C	T	C
46	GCC	GGC	TCC	TGC	AAA	TGC	AAA	GAT	TGC	AAG	TGC	GCC	TCC	TGC	AAG
16	P	G	S	C	K	C	K	D	C	R	C	A	S	C	K
91	AAG	AGC	TGC	TGC	TCC	TGC	TGC	CCC	GTG	GGC	TGT	GCC	AAG	TGT	GCC
31	K	S	C	C	S	C	C	P	V	G	C	A	K	C	A
136	CAG	GGC	TGC	GTC	TGC	AAA	GGG	GCT	TCG	GAC	AAG	TGC	AGC	TGC	TGC
46	Q	G	C	V	C	K	G	A	S	D	K	C	S	C	C
181	GCC														
61	A														

Figure 1. Open reading frames of, and AA sequences encoded by, metallothionein-I (MT-I) and metallothionein-II (MT-II) genes in the yak. The total open reading frame has a length of 183 nucleotides, representing a structurally intact protein of 61 AA, which includes 20 cysteines.

predicted by DNAMAN (http://www.lynnon.com/; last accessed Dec. 6, 2006), and tertiary structures of MT-I and MT-II were predicted by Swiss-Model (Peitsch, 1995; Guex and Peitsch, 1997; Schwede et al., 2003; http:// swissmodel.expasy.org//SWISS-MODEL.html; last accessed Dec. 6, 2006). The tertiary structure prediction of MT-II was graphed using Rasmol (http://www.u-mass.edu/microbio/rasmol/index2.htm; last accessed Dec. 6, 2006)

RESULTS AND DISCUSSION

Nucleotide and AA Sequences of MT-I and MT-II

The results of sequencing of MT-I and MT-II and the AA sequences encoded by MT-I and MT-II genes in the yak are given in Figure 1. Results of sequencing were consistent across numerous clones and showed that the amplified fragments were open reading frames of cDNA-

YAK. MT-1	MDPNCSCSTGGSCSCPGSCTCKACRCPSCKKSCCSCCPVG	40
COW. MT-1	MDPNCSCPTGSSCSCAGSCTCKACRCPSCKKSCCSCCPVG	40
MOUSE. MT-1	MDPNCSCSTGGSCTCTSSCACKNCKCTSCKKSCCSCCPVG	40
RABBIT. MT-1	MDPNCSCATGNSCTCASSCACKNCKCTSCKKSCCSCCPAG	40
PIG. MT-1	MDPNCSCSTGGSCSCAGSCTCKACRCTSCKKSCCSCCPAG	40
SHEEP. MT-1	MDPNCSCPTGSSCSCAGSCTCKACRCPSCKKSCCSCCPVG	40

YAK. MT-1	CAKCAQGCICKGASDKCSCCA	61
COW. MT-1	CAKCAQGCVCKGASDKCSCCA	61
MOUSE. MT-1	CSKCAQGCVCKGAADKCTCCA	61
RABBIT. MT-1	CTKCAQGCICKGASDKCSCCA	61
PIG. MT-1	CARCAQGCICKGASDKCSCCA	61
SHEEP. MT-1	CAKCAQGCVCKGASDKCSCCA	61

YAK. MT-2	MDPNCSCTAGESCTCAGSCKCKDCKCASCKKSCCSCCPVG	40
COW. MT-2	MDPNCSCTAGESCTCAGSCKCKDCKCASCKKSCCSCCPVG	40
MOUSE. MT-2	MDPNCSCASDGSCSCAGACKCKQCKCTSCKKSCCSCCPVG	40
RABBIT. MT-2	MDPNCSCATGNSCTCASSCKCKECKCTSCKKSCCSCCPAG	40
PIG. MT-2	MDPNCSCSTGGSCSCAGSCTCKACRCTSCKKSCCSCCPAG	40
SHEEP. MT-2	MDPNCSCTAGESCTCAGSCKCKDCKCASCKKSCCSCCPVG	40

YAK. MT-2	CAKCAQGCVCKGASDKCSCCA	61
COW. MT-2	CAKCAQGCVCKGASDKCSCCA	61
MOUSE. MT-2	CAKCSQGCICKEASDKCSCCA	61
RABBIT. MT-2	CTKCAQGCICKGASDKCSCCA	61
PIG. MT-2	CARCAQGCICKGASDKCSCCA	61
SHEEP. MT-2	CAKCAQGCVCKGASDKCSCCA	61

Figure 2. Analysis of AA sequences of yak, cow, sheep, mouse, rabbit, and pig metallothionein (MT). The tripeptides conserved across species (and enclosed in the boxes) are: MDPN CXC—CXC—CXC—CXC—C—CCXCC—CXXC—CXC—CXCC—, where X designates AA of the MT-I and MT-II excluding cysteines. GenBank Accession No.: yak MT-I (AY513744); cow MT-I (BTA489255); rabbit MT-I (X07790); pig MT-I (NM_001001266); sheep MT-I (X00953); yak MT-II (AY513745); cow MT-II (BTA489256); mouse MT-II (AY341879); rabbit MT-II (X07791); and pig MT mRNA (AB000794).

Table 2. Analysis of protein signal peptides with the SignalP 3.0 Server[1]

Prediction models	SignalP-NN[2]			SignalP-HMM[2]			Conclusions	
	Scores[3]	Value	Cutoff	Signal peptide probability	Signal anchor probability	Max cleavage site probability	Prediction	Signal peptide
MT-I	C	0.103	0.32	0.026	0.000	0.016	Nonsecretory protein	No
	S	0.039	0.33					
	Y	0.305	0.87					
MT-II	C	0.212	0.32	0.004	0.000	0.002	Nonsecretory protein	No
	S	0.032	0.33					
	Y	0.231	0.87					

[1]http://www.cbs.dtu.dk/services/SignalP-3.0/; last accessed Dec. 6, 2006.
[2]NN = neural networks; HMM = hidden Markov models.
[3]C = raw cleavage site score; S = signal peptide score; Y = combined cleavage site score.

encoding MT-I and MT-II of the yak, each with a length of 183 bp (Genbank Accession Nos. AY513744 and AY513745). Metallothioneins have been cloned in other mammals, namely, mouse (*M. musculus*) MT-I (NM_013602) and MT-II (NM_008630), sheep (*O. aries*) MT-I (X04626) and MT-II (X07975), and rabbit (*O. cuniculus*) MT-I (X07790) and MT-II (X07791). A BLAST search of the cDNA sequences of MT-I and MT-II indicated that the MT-I coding sequences of the sheep, cow, pig, and human shared 96, 95, 93, and 89% sequence similarity, respectively, with the yak. The MT-II coding sequences of the cow, human, mouse, and pig shared 97, 92, 92, and 91% sequence similarity, respectively, with the yak. Multiple alignments performed using the BioEdit program showed that the nucleotide sequences of MT-I and MT-II of the yak between different species were highly conservative. The 2 isoforms, MT-I and MT-II, are ubiquitously expressed in all mammalian tissues (Aschner, 1996). However, there is little sequence homology (~35%) between invertebrate MT and mammalian MT. Mammalian MT-I and MT-II, on the other hand, share high sequence identity (~85%; Zangger et al., 1999).

The yak cDNA nucleotide sequences were translated into proteins containing 61 AA, which include 20 cysteines. These results agree with work in the mouse (Durnam et al., 1980; Beach and Palmiter, 1981; Searle et al., 1984; Stallings et al., 1984), sheep (Peterson and Mercer, 1986; Peterson et al., 1988), and rabbit (Tam et al., 1988), for whom MT proteins with 61 AA and 20 cysteines have been reported. Comparisons of the AA sequence of MT-I with MT-II of the yak indicated several differences between Ser[8], Thr[9], Gly[11], Ser[14], Pro[16], Thr[20], Ala[23], Arg[25], Pro[27], and Ile[49] in MT-I sequences and Thr[8], Ala[9], Glu[11], Thr[14], Ala[16], Lys[20], Asp[23], Lys[25], Ala[27], and Val[49] in MT-II sequences, with superscripts denoting AA residue sites in the MT-I and MT-II sequences.

Comparisons of AA sequences of MT-I and MT-II in the yak with conserved sequences in the cow, sheep, mouse, rabbit, and pig showed many conserved tripeptides, such as C-X-C, C-C-X-C-C, and C-X-X-C, which are highly conserved in their evolution and derivation (Figure 2).

Characterization of the MT-I and MT-II Proteins

Analysis of the molecular weight and AA composition of the MT-I and MT-II performed using BioEdit showed that the molecular weights of yak MT-I and MT-II were 5980.87 Da and 6027.93 Da, respectively. Analyses also indicated that they exhibited an unusual AA composition in that 1) they had no aromatic AA; 2) they exhibited a single polypeptide chain of 61 AA; and 3) 33% of their residues were cysteines (20 residues in mammalian MT at invariant positions).
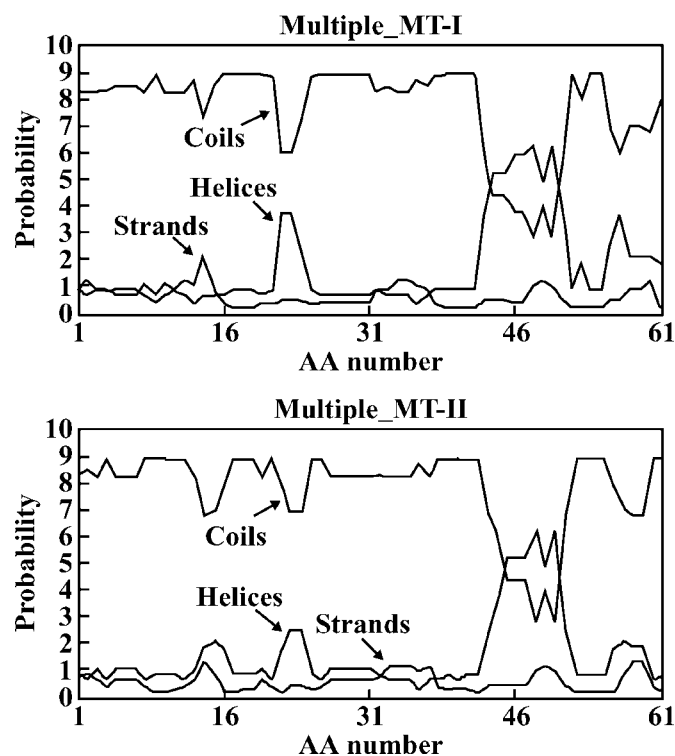

Figure 3. Prediction of the secondary structure of metallothionein-I (MT-I) and metallothionein-II (MT-II) of the yak and other mammals. There are obvious coils and no obvious strands in the MT-I and MT-II protein structures, and there are obvious helices in the 42nd to 54th AA.
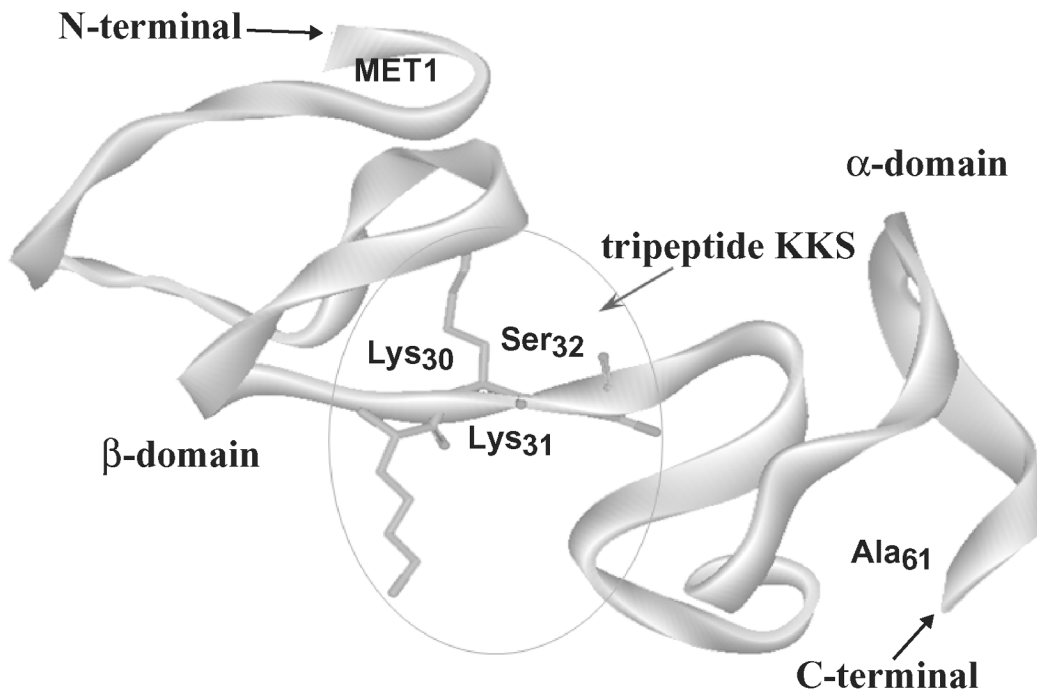
Figure 4. Model of the tertiary structure of yak MT-II from SwissProt (Strands model). The prediction parameters were as follows: BLAST limit = 0.00001; template choice = default BLAST search in ExPDB; result option = normal mode. There were 2 domains, the N-terminal β-domain and the C-terminal α-domain (shown), which were linked by the tripeptide Lys^{30}-Lys^{31}-Ser^{32} (KKS).

Hydrophobicity, Transmembrane Region, and Physiological Function

Analyses of hydrophocity and transmembrane regions indicated there were no obvious hydrophobic domains or transmembrane regions, suggesting that the proteins were probably not transmembrane proteins. Results from the protein signal peptide analysis suggested that MT-I and MT-II proteins were nonsecretory cytoplasmic proteins (Table 2).

Metallothionein-I and MT-II positive cells are known to be present mainly in astrocytes but not in oligodedroglia, neuroglia, or neurons (Ono et al., 1997). Metallothionein is implicated in many neurological diseases, including Alzheimer's disease (Zambenedetti et al., 1998), epilepsy (Erickson et al., 1997), brain anoxemia (Neal et al., 1996), amyotrophic lateral sclerosis (Sillevis et al., 1994; Blaauwgeers et al., 1996), and brain tumors (Nagane et al., 1995; Maier et al., 1997).

Structure Prediction of the MT-I and MT-II Proteins

Secondary structure prediction of the MT-I and MT-II from the DNAMAN analysis indicated similarities among the yak and other mammals in their protein structures (Figure 3). A graph of the tertiary structure prediction of the yak MT-II is shown in Figure 4.

Metallothionein genes have been cloned from several genera and species, including the human, cow, sheep, pig, rabbit, and now the yak. Studies have demonstrated that the structures of MT genes are highly similar in mammals, having 3 exons and 2 introns (Hamer, 1986; West et al., 1990; Hudspeth et al., 1996), and that these coding sequences are highly conservative. Therefore, the gene-specific primers for RT-PCR in this study were designed for homological comparison of the mammalian genes, and the coding sequences of MT-I and MT-II in the yak were cloned by RT-PCR using these primers. The results of MT-I and MT-II sequencing suggest that the full-length coding sequences of MT-I and MT-II are 183 bp in the yak, in which specific initiation and terminal codons reside. Analysis of the characterization and structure indicated that MT of the yak are highly conservative, low-molecular-weight (approximately 6,000 Da), cysteine-rich metal-binding nonsecretory cytoplasmic proteins in which there are no obvious hydrophobic domains, transmembrane regions, or signal peptides. The key structure of yak liver MT includes 2 domains, α and β, and the presence of the 2 well-defined domains results in formation of an overall dumbbell shape of the metalloprotein. The N-terminal β-domain consists of residues 1 to 30, and the C-terminal α-domain consists of residues 31 to 62. The domains are linked by tripeptide Lys^{30}-Lys^{31}-Ser^{32} (KKS).

In conclusion, heavy metals, hormones, inflammation, acute stress, and many chemicals can induce activity in 2 isoforms of the metallothionein multigene family (metallothionein I and II). Yak (*Bos grunniens*) represents a unique bovine species adapted to the Qinghai-

Tibetan plateau of China at altitudes of 3,000 m above sea level. There is a need to evaluate the isoforms of metallothioneins in this species to determine potential tolerance to heavy metal contaminants. Metallothionein-I and II coding sequences of the sheep, cow, pig, and human shared high sequence similarity with the yak. Thus, tolerances to heavy metal contamination are expected to parallel those of other species. However, there is a need for further study of metallothionein III and IV to better understand the characteristics of metallothionein activity in the yak.

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